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Single-Stranded Antisense siRNAs Guide Target RNA Cleavage in RNAi

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Summary

Small interfering RNAs (siRNAs) are the mediators of mRNA degradation in the process of RNA interference (RNAi). Here, we describe a human biochemical system that recapitulates siRNA-mediated target RNA degradation. By using affinity-tagged siRNAs, we demonstrate that a single-stranded siRNA resides in the RNA-induced silencing complex (RISC) together with eIF2C1 and/or eIF2C2 (human GERp95) Argonaute proteins. RISC is rapidly formed in HeLa cell cytoplasmic extract supplemented with 21 nt siRNA duplexes, but also by adding single-stranded antisense RNAs, which range in size between 19 and 29 nucleotides. Single-stranded antisense siRNAs are also effectively silencing genes in HeLa cells, especially when 5′-phosphorylated, and expand the repertoire of RNA reagents suitable for gene targeting.

Introduction

Most eukaryotes possess a cellular defense system protecting their genomes against invading foreign genetic elements. Insertion of foreign elements is believed to be generally accompanied by formation of dsRNA that is interpreted by the cell as a signal for unwanted gene activity (Fire et al., 1998; Ketting et al., 1999; Tabara et al., 1999; Waterhouse et al., 2001; Ahlquist, 2002; Plasterk, 2002). Dicer RNase III rapidly processes dsRNA to small dsRNA fragments of distinct size and structure (Bernstein et al., 2001; Billy et al., 2001; Ketting et al., 2001), the small interfering RNAs (siRNAs) (Elbashir et al., 2001b), which direct the sequence-specific degradation of the single-stranded mRNAs of the invading genes (Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001b). siRNA duplexes have 2 to 3 nt 3′ overhanging ends and contain 5′ phosphate and free 3′ hydroxyl termini (Elbashir et al., 2001b, 2001c; Zamore et al., 2000). The process of posttranscriptional dsRNA-dependent gene silencing is commonly referred to as RNA interference (RNAi) (for recent reviews, see Hannon, 2002; Zamore, 2002; Matzke et al., 2001; Sharp, 2001; Tuschl, 2001).

Experimental introduction of siRNA duplexes into mammalian cells is now widely used to disrupt the activity of cellular genes homologous in sequence to the introduced dsRNA. Used as a reverse genetic approach, siRNA-induced gene silencing accelerates linking of gene sequence to biological function (Elbashir et al., 2001a, 2002; Harborth et al., 2001). siRNA duplexes are short enough to bypass general dsRNA-induced unspecific effects in vertebrate animal and mammalian cells (Elbashir et al., 2001a; Bitko and Barik, 2001). siRNAs may also be expressed intracellularly from introduced expression plasmids or viral vectors providing an alternative to chemical RNA synthesis (for a short review, see Tuschl, 2002). An understanding of how siRNAs act in mammalian systems is important for refining this gene silencing technology and for producing gene-specific therapeutic agents (Tuschl and Borkhardt, 2002).

Biochemical studies have begun to unravel the mechanistic details of RNAi. The first cell-free systems were developed using *D. melanogaster* cell or embryo extracts (Hammond et al., 2000; Tuschl et al., 1999; Zamore et al., 2000) and were followed by the development of in vitro systems from *C. elegans* embryo (Ketting et al., 2001) and mouse embryonal carcinoma cells (Billy et al., 2001). While the *D. melanogaster* lysates support the steps of dsRNA processing and sequence-specific mRNA targeting, the latter two systems recapitulate the first step only.

RNAi in *D. melanogaster* extracts is initiated by ATP-dependent processing of long dsRNA to siRNAs by Dicer RNase III (Zamore et al., 2000; Bernstein et al., 2001; Ketting et al., 2001; Zamore, 2001). Thereafter, siRNA duplexes are assembled into a multi-component complex, which guides the sequence-specific recognition of the target mRNA and catalyzes its cleavage (Hammond et al., 2000; Yang et al., 2000; Zamore et al., 2000; Elbashir et al., 2001b). This complex is referred to as RNA-induced silencing complex (RISC; Hammond et al., 2000). siRNAs in *D. melanogaster* are predominantly 21 and 22 nt (Elbashir et al., 2001b), and when paired in a manner to contain a 2 nt 3′ overhang, effectively enter RISC (Elbashir et al., 2001c). Mammalian systems have siRNAs of similar size (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002), and siRNAs of 21 and 22 nt also effectively silence genes in mammalian cells (Caplen et al., 2001; Elbashir et al., 2001a, 2002).

RISC assembled on siRNA duplexes in *D. melanogaster* embryo lysate targets homologous sense as well as antisense single-stranded RNAs for degradation (Elbashir et al., 2001b, 2001c). The cleavage sites for sense and antisense target RNAs are located in the middle of the region spanned by the siRNA duplex. The 5′ end, and not the 3′ end, of the guide siRNA sets the ruler for the position of the target RNA cleavage (Elbashir et al., 2001b, 2001c). A 5′ phosphate is required at the target-complementary strand of a siRNA duplex for RISC activity, and ATP is used to maintain the 5′ phosphates of the siRNAs (Nykänen et al., 2001). Synthetic siRNA duplexes with free 5′ hydroxyls and 2 nt 3′ overhangs are rapidly phosphorylated in *D. melanogaster* embryo lysate, so that the RNAi efficiencies of 5′-phosphorylated and non-phosphorylated siRNAs are not significantly different (Elbashir et al., 2001c).

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Unwinding of the siRNA duplex must occur prior to target RNA recognition. Analysis of ATP requirements revealed that the formation of RISC on siRNA duplexes required ATP in lysates of *D. melanogaster* (Nykänen et al., 2001). Once formed, RISC cleaves the target RNA in the absence of ATP (Hammond et al., 2000; Nykänen et al., 2001). The need for ATP probably reflects the unwinding step and/or other conformational rearrangements. However, it is currently unknown if the unwound strands of a siRNA duplex remain associated with RISC or whether RISC only contains a single-stranded siRNA. The symmetric cleavage of sense and antisense target RNA by siRNA duplexes (Elbashir et al., 2001b, 2001c) may be explained by the presence of approximately equal populations of sense and antisense strand-containing RISCs.

A component associated with RISC was identified as Argonaute2 from *D. melanogaster Schneider 2* (S2) cells (Hammond et al., 2001), and it is a member of a large family of proteins. The family is referred to as Argonaute or PPD family and is characterized by the presence of a PAZ domain and a C-terminal Piwi domain, both of unknown function (Cerutti et al., 2000; Schwarz and Zamore, 2002). The PAZ domain is also found in Dicer (Cerutti et al., 2000). Because Dicer and Argonaute2 interact in S2 cells, PAZ may function as a protein-protein interaction motif (Hammond et al., 2001). Possibly, the interaction between Dicer and Argonaute2 facilitates siRNA incorporation into RISC. The mammalian members of the Argonaute family are poorly characterized, and some of them have been implicated in translational control, microRNA processing, and development (Zou et al., 1998; Sharma et al., 2001; Deng and Lin, 2002; Mourelatos et al., 2002). The biochemical function of Argonaute proteins remains to be established and the development of more biochemical systems is crucial.

Here, we report on the analysis of human RISC in extract prepared from HeLa cells. The reconstitution of RISC and the mRNA targeting step were exploited for affinity purification of RISC and revealed that RISC is a ribonucleoprotein complex that contains a single-stranded siRNA and proteins of the Argonaute family. Once RISC is formed, the incorporated siRNA can no longer exchange with free siRNAs. Surprisingly, RISC can be reconstituted in HeLa S100 extract providing single-stranded siRNAs. Even more surprising, single-stranded antisense siRNAs transfected into HeLa cells potently silence an endogenous gene with similar efficiency to duplex siRNA. Dissecting the individual mechanistic steps of RNAi in mammalian systems will help to modify the properties of siRNAs and to facilitate prediction of mRNA targeting efficiency as a tool for validation of therapeutic targets or even as gene-specific therapeutic itself.

Results

A Human Biochemical System for siRNA Functional Analysis

To investigate whether siRNAs guide target RNA degradation in human cells by a similar mechanism to the one observed in *D. melanogaster* (Elbashir et al., 2001b, 2001c), we prepared substrates for targeted mRNA degradation as described previously (Elbashir et al., 2001c). A 5′-^{32}P-cap-labeled, 177 nt RNA transcript, derived from a segment of the firefly luciferase gene, was incubated in HeLa cell S100 or *D. melanogaster* embryo extracts with a 21 nt siRNA duplex in the presence of an ATP regeneration system (Figures 1A and 1B). siRNA cleavage assays were performed at 25°C in *D. melanogaster* lysate and at 30°C in HeLa S100 extract for 2.5 hr. After deproteinization using proteinase K, the reaction products were separated on a 6% sequencing gel.

Similar to the previous observation in *D. melanogaster* lysate, we observed the appearance of a cleavage product in cytoplasmic HeLa S100 extract at exactly the same position, thus indicating that the siRNA duplex guides target RNA cleavage in the human system with the same specificity and mechanism (Figure 1B); nuclear extract assayed under the same conditions did not support siRNA-specific target RNA cleavage (data not shown). The cleavage reaction appeared less efficient when compared to the *D. melanogaster* system, but this could be explained by the 5-fold lower total protein concentration of HeLa S100 extract (25 mg/ml versus 5 mg/ml). Similar to *D. melanogaster* lysates, siRNA duplexes without 5′ phosphate were rapidly 5′-phosphorylated in HeLa S100 extract (see below) and the ability to cleave the target RNA was independent of the presence of a 5′ phosphate on the synthetic siRNA duplexes.

Comparative analysis of the efficiency of siRNA duplexes of different length in *D. melanogaster* lysate and in transfected mammalian cells indicated that the differences in silencing efficiencies between 20 to 25 nt siRNA duplexes were less pronounced in mammalian cells than in *D. melanogaster* (Caplen et al., 2001; Elbashir et al., 2002). Duplexes of 24 and 25 nt siRNAs were inactive in *D. melanogaster* lysate, whereas the same duplexes were quite effective for silencing when introduced by transfection into HeLa cells (Elbashir et al., 2002). We therefore asked whether siRNA duplexes of 20 to 25 nt are able to reconstitute RISC with similar efficiency. No considerable differences were observed in our biochemical assay (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/110/5/563/DC1). A single target RNA cleavage site was mapped for all duplexes, and the position of target RNA cleavage was defined relative to the 5′ end of the antisense strand of the siRNA duplexes predicted by the cleavage guiding rules established in *D. melanogaster* lysate (Elbashir et al., 2001c). This suggests that siRNA duplexes slightly larger than the natural 21 nt siRNA duplexes may directly enter RISC without further processing by Dicer.

5′ Modification of the Guide siRNA Inhibits RISC Activity

Modification of siRNAs at their termini is important for developing siRNA-based affinity purification schemes or for conjugating reporter tags for biophysical measurements. The most common method for introducing reactive side chains into nucleic acids is chemical synthesis using aminolinker derivatives (Eckstein, 1991). We have introduced 5′ and 3′ aminolinkers with six and seven methylene groups as spacers, respectively. The linker-modified siRNA duplexes were tested for mediating target RNA degradation in HeLa S100 extract (Supplemen-

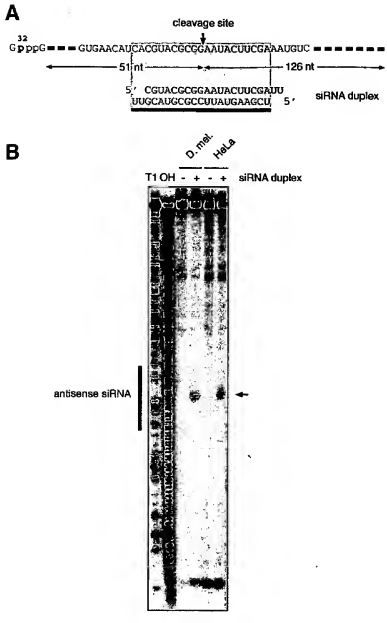

Figure 1. HeLa Cytoplasmic S100 Extract Show siRNA-Dependent Target RNA Cleavage

(A) Representation of the 177 nt ^{32}P-cap-labeled target RNA with the targeting siRNA duplex. Target RNA cleavage site and the length of the expected cleavage products is also shown. The fat black line positioned under the antisense siRNA is used in the following figures as symbol to indicate the region of the target RNA, which is complementary to the antisense siRNA sequence.

(B) Comparison of the siRNA mediated target RNA cleavage using the previously established *D. melanogaster* embryo in vitro system and HeLa cell S100 cytoplasmic extract. 10 nM cap-labeled target RNA was incubated with 100 nM siRNA as described in materials. Reaction products were resolved on a 6% sequencing gel. Position markers were generated by partial RNase T1 digestion (T1) and partial alkaline hydrolysis (OH) of the cap-labeled target RNA. The arrow indicates the 5′ cleavage product; the 3′ fragment is unlabeled and therefore invisible.

tal Figure S2 available at http://www.cell.com/cgi/content/full/110/5/563/DC1). Modification of the 5′ end of the antisense guide siRNA abolished target RNA cleavage, while modification of neither the sense 5′ end nor of both 3′ ends showed any inhibitory effect. In an identical experiment using *D. melanogaster* embryo lysate, we observed a similar pattern of RISC activity although the duplex carrying the 5′ aminolinker-modified antisense siRNA showed some residual activity (data not shown). Presumably, introduction of additional atoms or the change in terminal phosphate electric charge at the 5′ end of the antisense siRNA interfered with its ability to function as guide RNA. The critical function of the guide siRNAs 5′ end was previously documented (Nykänen et al., 2001; Elbashir et al., 2001c).

The ability to modify siRNAs at their 3′ end suggests that siRNAs need not act as primers for degradative PCR (Lipardi et al., 2001). The fate of siRNAs in HeLa S100 extract was followed by incubation of siRNA duplexes, in which the antisense siRNA was ^{32}pCp-radiolabeled and contained various 5′ and 3′ hydroxyl/phosphate modifications (Figure 2A). All combinations of duplex siRNAs were fully competent for RISC-dependent target RNA degradation (data not shown). As previously observed for *D. melanogaster* lysates (Nykänen et al., 2001), rapid 5′ phosphorylation of siRNA duplexes with free 5′ hydroxyl termini was detected in HeLa S100 extract (Figure 2B). To our surprise, we noted that a small fraction of the 3′-phosphorylated antisense siRNA was ligated to the opposing 5′ hydroxyl of the sense siRNA producing a lower mobility band. The inter-strand ligation was confirmed by changing the length of the unlabeled sense siRNA, which resulted in the expected mobility changes of the ligation product (data not

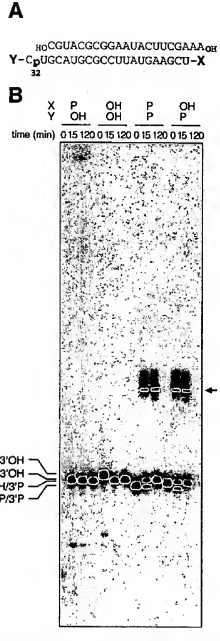

Figure 2. siRNAs Containing 3′-Terminal Phosphates Are Subjected to Ligation as well as Dephosphorylation Reactions

(A) Sequence of the radiolabeled siRNA duplex. The labeled nucleotide was joined to synthetic 20 nt antisense siRNA by T4 RNA ligation of ^{32}pCp. The various combinations of 5′ and 3′ hydroxyl/phosphate were prepared as described in materials. X and Y indicate 5′ and 3′ modifications of the antisense siRNA.

(B) Fate of the antisense siRNA during incubation of the modified siRNA duplexes in HeLa S100 extract in the presence of nonradiolabeled target RNA. The different phosphorylated forms of the antisense siRNA were distinguished based on their gel mobility. Identical results were obtained when using 5′-phosphorylated sense siRNA or when leaving out the target RNA during incubation. Ligation products are only observed when 3′ phosphates were present on the labeled antisense siRNA.

shown). RNA ligase activity was previously observed in HeLa S100 extract and is mediated by two enzymatic activities (Genschik et al., 1997). The 3′ terminal phosphate is first converted to a 2′,3′-cyclic phosphate requiring ATP and 3′ terminal phosphate cyclase. Thereafter, the opposing 5′ hydroxyl is ligated to the cyclic phosphate end by an as yet uncharacterized RNA ligase, but most likely tRNA ligase (Filipowicz and Shatkin, 1983). We chemically synthesized the predicted 5′-phosphorylated, 42 nt ligation product and found it unable to mediate target RNA cleavage (data not shown), presumably because it cannot form activated RISC. The majority of the 3′- phosphorylated siRNA duplexes was gradually dephosphorylated at its 3′ end and emerged chemically similar to naturally generated siRNA. Together, these observations indicate that the cell has a mechanism to preserve the integrity of siRNAs. We were unable to detect proposed siRNA-primed polymerization products (Figure 2B), suggesting that siRNAs do not function as primers for template-dependent dsRNA synthesis in our system. However, we acknowledge that a proposed RNA-dependent polymerase activity may have been inactivated during preparation of our extract.

siRNAs Incorporated into RISC Do Not Compete with a Pool of Free siRNAs

In order to analyze RISC assembly and stability, we tested whether a target-unspecific siRNA duplex, was able to compete with a target-specific siRNA duplex. The unspecific siRNA duplex was directed against the *Renilla reniformis* luciferase sequence (Elbashir et al., 2001a). When specific and nonspecific siRNA duplexes were coincubated in HeLa S100 extract, increasing concentrations of unspecific siRNA duplex competed with the formation of target-specific RISC (Supplemental Figure S3 available at http://www.cell.com/cgi/content/full/110/5/563/DC1). However, when target-specific siRNAs were preincubated in HeLa S100 extract for 15 min in the absence of competitor siRNA duplex, the assembled siRNA in the target-specific RISC could no longer be competed with the target-unspecific siRNA duplex. In both cases, the cap-labeled target RNA was added 15 min after the addition of the competitor siRNA duplex. This result suggests that RISC is formed during the first 15 min of incubation and that siRNAs were irreversibly associated with the protein components of RISC during the 2.5 hr time window of the experiment.

Partial Purification of Human RISC

After having defined the 3′ termini of siRNAs as the most suitable position for chemical modification, a photocleavable biotin derivative was conjugated to the 3′ aminolinker-modified siRNAs. A photo-cleavable biotin derivative was selected because of the advantage of recovering RISC under nondenaturing conditions after capturing complexes on streptavidin-coated affinity supports. Conjugation of photo-cleavable biotin to the 3′ and of sense, antisense, or to both of the strands did not affect target RNA cleavage when compared to nonbiotinylated siRNAs (data not shown). siRNA duplexes with biotin residues on both 3′ ends were used for affinity purification (Figure 3A). The double biotinylated siRNA duplex was incubated in HeLa S100 extract in the

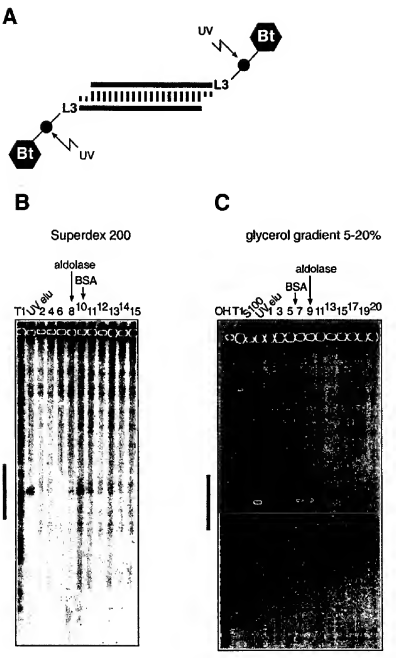

Figure 3. Partial Purification of Human RISC

(A) Graphical representation of the structure of the biotinylated siRNA duplex used for affinity purification of siRNA-associated factors. L3 indicates a C7-aminolinker that was conjugated to a photo-cleavable biotin N-hydroxysuccinimidyl ester; UV indicates photocleavage of the UV-sensitive linkage to release affinity-selected complexes under native conditions.

(B) Superdex-200 gel filtration analysis of siRNA-protein complexes (siRNPs) recovered by UV treatment/elution (UV elu) from the streptavidin affinity column. Fractions were assayed for their ability to sequence specifically cleave the cap-labeled target RNA. The number of the collected fractions and the relative positions of the aldolase (158 kDa) and BSA (66 kDa) size markers are indicated.

(C) Glycerol gradient (5%–20%) sedimentation of siRNPs recovered by UV treatment/elution from the streptavidin affinity column. For legend, see (B). When monitoring the precise size of target RNA cleavage fragments using internally ^{32}P-UTP-labeled, capped mRNA, the sum is equal to the full-length transcript, thus indicating that target RNA is indeed only cleaved once in the middle of the region spanned by the siRNA.

presence of ATP, GTP, creatine phosphate, and creatine kinase for ATP regeneration. Thereafter, streptavidin-conjugated agarose beads were added to capture the biotinylated siRNA ribonucleoprotein complexes (siRNPs) including RISC. After extensive washing of the collected beads, the siRNPs were released by UV irradiation at 312 nm. The eluate cleaved target RNA sequence specifically, thus indicating that RISC was recovered in its native state from the resin (Figures 3B, 3C, and lane UV elu). The flowthrough from the affinity selection showed no detectable RISC activity indicating complete binding of RISC by the beads. It was also possible to affinity select RISC upon reconstitution with single-stranded, 3′ biotinylated antisense siRNA, although with lower efficiency (data not shown). Affinity eluates were further analyzed by gel filtration on Superdex 200 columns (Figure 3B) as well as 5%–20% glycerol gradient ultra-centrifugation (Figure 3C). Individual fractions were collected and assayed for target RNA cleavage without addition of any further siRNA. RISC activity fractionated closer to the molecular size marker aldolase (158 kDa) than to BSA (66 kDa), after gel filtration or glycerol gradient centrifugation (Figures 3B and 3C). The molecular size of human RISC is therefore estimated to be between 90 and 160 kDa, and smaller than the complexes previously described in *D. melanogaster* lysates (Hammond et al., 2000; Nykänen et al., 2001). Using the same methods for analysis of reconstituted RISC in *D. melanogaster* S2 cell cytoplasmic extract, we estimated a molecular size similar to the human RISC (data not shown).

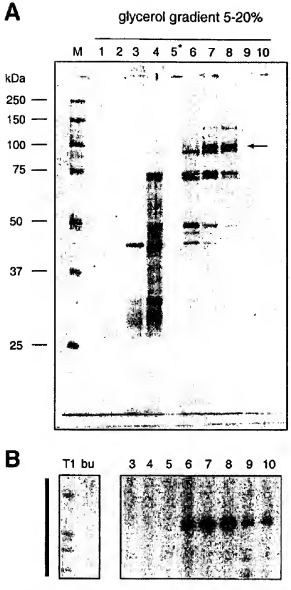

Figure 4. Protein Composition of Affinity Purified RISC

(A) Silver-stained SDS-PAGE gel of affinity-selected ribonucleoprotein complexes after glycerol gradient (5%–20%) sedimentation. The arrow indicates the band containing eIF2C1 and eIF2C2. Molecular size markers are indicated on the left. The asterisk indicates a fraction for which the protein pellet was lost after precipitation.

(B) Target RNA cleavage assay of the collected fractions. RISC activity peaked in fraction 7 and 8; bu, buffer.

Two proteins of approximately 100 kDa were identified by mass spectrometry in the peak fraction of the endonucleolytic activity of the 5%–20% glycerol gradient (Figure 4, fractions 7 and 8), corresponding to eIF2C1 and eIF2C2/GERp95 (Supplemental Figure S4 available at http://www.cell.com/cgi/content/full/110/5/563/DC1). The proteins are 82% similar and are both members of the Argonaute family. One of its members, Argonaute2, was previously identified as a component of *D. melanogaster* RISC (Hammond et al., 2001). The proteins present within the other bands remain to be identified.

The absence of a 210 kDa protein band in the affinity purified fraction, and also the relatively small observed size of RISC suggests that Dicer is not present in RISC. To test whether Dicer may be involved in the formation of RISC on synthetic siRNAs, we immunodepleted HeLa S100 extract using affinity purified anti-peptide sera against human Dicer (generously provided by E. Billy and W. Filipowicz). Efficient immunodepletion was confirmed by Western blotting (Supplemental Figure S5 available at http://www.cell.com/cgi/content/full/110/5/563/DC1). The supernatant retained the ability for targeting siRNA-directed target RNA cleavage, while the resuspended immunoprecipitate was inactive (Supplemental Figure S5 available at above URL).

RISC Contains a Single siRNA Strand and Can Be Reconstituted Using Single-Stranded siRNAs

To address the constitution of siRNA strands in RISC, we affinity selected the assembled complexes using siRNA duplexes that were biotinylated at only one of the two constituting strands or both. If sense and antisense strands were present together in RISC, the cleavage activity should be affinity selected on Neutravidin independently of the position of the biotin residue. In contrast, we observed target RNA cleavage from UV eluates after streptavidin selection only for siRNA duplexes with biotin conjugated to the antisense strand, but not to the sense strand (Figure 5). RISC activity, assembled on siRNA duplexes with only the sense siRNA biotinylated, remained in the flowthrough. These data suggest that RISC contains only a single-stranded RNA molecule.

To assess whether single-stranded siRNA may be able to reconstitute RISC, single-stranded siRNA as well as a siRNA duplex were incubated at concentrations between 1 to 100 nM with cap-labeled target RNA in HeLa S100 as well as *D. melanogaster* extracts (Figure 6). At 100 nM single-stranded antisense siRNA, we clearly detected RISC-specific target RNA cleavage in HeLa extract, thus confirming that single-stranded siRNA is present in RISC. At lower concentrations of single-stranded siRNA, RISC formation remained undetectable while duplex siRNA was effectively forming RISC even at 1 nM concentration. Therefore, a specific pathway exists which converts double-stranded siRNA into single-stranded siRNA containing RISC. In *D. melanogaster* embryo lysate, we were unable to detect RISC activity from antisense siRNA, presumably because of the high load of single-strand specific ribonucleases (Elbashir et al., 2001b). We then assessed the length requirements for reconstituting RISC and used 5′-phosphorylated single-stranded antisense siRNAs varying in length between 13 to 29 nts. siRNAs between 15 to 29 nt mediated RISC-specific target RNA degradation in HeLa S100 extract, and cleavage efficiency increased with increasing siRNA length (Supplemental Figure S6 available at http://www.cell.com/cgi/content/full/110/5/563/DC1). Reconstitution of RISC was also accomplished using nonphosphorylated single-stranded antisense siRNAs (data not shown), but presumably, 5′ phosphorylation of single-stranded siRNAs occurred during the incubation in the S100 extract.

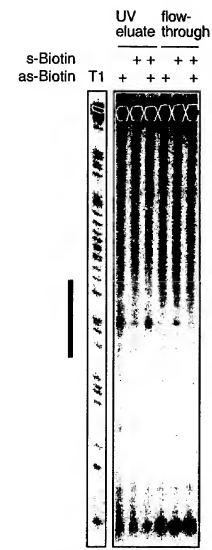

Figure 5. RISC Contains a Single-Stranded siRNA

siRNPs were subjected to affinity selection after incubation using siRNA duplexes with one or both strands biotinylated. The eluate recovered after UV treatment or the unbound fraction after streptavidin affinity selection (flowthrough) was assayed for target RNA degradation. If the antisense strand was biotinylated, all sense target RNA-cleaving RISC was bound to the streptavidin beads, while sense siRNA biotinylation resulted in RISC activity of the flowthrough. The cleavage reaction in the flowthrough fraction was less efficient than in the UV eluate, because affinity-selected RISC was more concentrated.

5′-Phosphorylated Antisense siRNAs Silence Endogenous Genes in Cultured Cells

Single-stranded 5′ hydroxyl and 5′ phosphate-modified antisense siRNAs were also tested for targeting of an endogenous gene in HeLa cells following the standard transfection protocol previously established for silencing of lamin A/C (Elbashir et al., 2001a, 2002). 200 nM concentrations of single-stranded siRNAs or 100 nM concentrations of duplex siRNAs were transfected into HeLa cells. Lamin A/C levels were monitored 48 hr later using immunofluorescence (Figure 7A) as well as quantitative luminescence-based Western blot analysis (Figure 7B). Gene silencing was observed with phosphorylated as well as nonphosphorylated antisense siRNAs ranging in size between 19 to 29 nt. The phosphorylated antisense siRNAs were consistently better performing than the nonphosphorylated antisense, and their silencing efficiencies were comparable to that of the conventional duplex siRNA.

Discussion

The development of a human biochemical system for analysis of the mechanism of RNAi is important given the recent success of siRNA duplexes for silencing genes expressed in human cultured cells and the potential for becoming a sequence-specific therapeutic agent (Elbashir et al., 2002; Harborth et al., 2001; Novina et al., 2002; Tuschl and Borkhardt, 2002). Biochemical systems are useful for defining the individual steps of the RNAi process and for evaluating the constitution and molecular requirements of the participating macromolecular complexes. For the analysis of RNAi, several systems were developed (Tuschl et al., 1999; Zamore et al., 2000; Hammond et al., 2000; Billy et al., 2001; Ketting et al., 2001). For mammalian systems, reconstitution of the mRNA targeting reaction was not yet reported. Here, we describe the development and application of a biochemical system prepared from the cytoplasmic fraction of human HeLa cells, which is able to reconstitute the mRNA-targeting RNA-induced silencing complex (RISC) (Hammond et al., 2000). Formation of RISC was accomplished using either 5′-phosphorylated or 5′ hydroxyl siRNA duplexes as well as single-stranded antisense siRNAs. Nonphosphorylated siRNA duplexes are rapidly 5′-phosphorylated in HeLa cell extract.

Biochemical Characterization of siRNA Function

Reconstitution of RISC activity was observed using cytoplasmic HeLa extract, but not nuclear extract, suggesting that RISC components are located predominantly in the cytoplasm. Cytoplasmic localization of the RNAi machinery is also supported by the observation that Dicer is predominantly cytoplasmic (Billy et al., 2001) and that RNAi acts on mature rather than nuclear precursor mRNA (Montgomery et al., 1998).

It has previously been proposed that siRNAs act as primers for target RNA-templated dsRNA synthesis (Lipardi et al., 2001), even though homologs of the RNA-dependent RNA polymerases known to participate in gene silencing in other systems are apparently not encoded in *D. melanogaster* or mammalian genomes. Analysis of the fate of siRNA duplexes in the HeLa cell system did not provide evidence for such a siRNA-primed activity, but indicates that the predominant pathway for siRNA-mediated gene silencing is sequence-specific endonucleolytic target RNA degradation. Further evidence against siRNA-induced propagation of gene silencing in mammalian systems is that (1) the silenced gene returns to normal levels between 5 to 9 days posttransfection (Elbashir et al., 2002); (2) simultaneously expressed isoforms can be selectively targeted by siRNA duplexes (Kisielow et al., 2002).

By using two independent methods, it was shown that

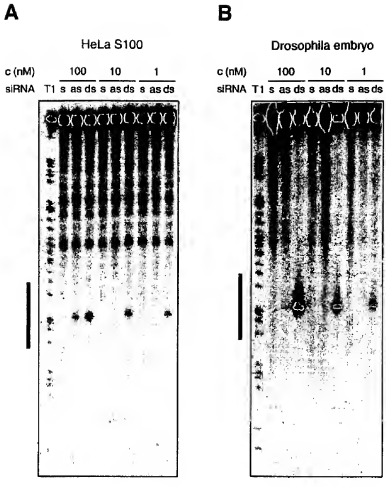

Figure 6. Single-Stranded Antisense siRNAs Reconstitute RISC in HeLa S100 Extract

Analysis of RISC reconstitution using single-stranded or duplex siRNAs comparing HeLa S100 extract (A) and the previously described *D. melanogaster* embryo lysate (B). Different concentrations of single-stranded siRNAs (s, sense; as, antisense) and duplex siRNA (ds) were tested for specific targeting of cap-labeled substrate RNA. 100 nM concentrations of the antisense siRNA reconstituted RISC in HeLa S100 extract, although at reduced levels in comparison to the duplex siRNA. Reconstitution with single-stranded siRNAs was almost undetectable in *D. melanogaster* lysate, presumably because of the higher nuclease activity in this lysate causing rapid degradation of uncapped single-stranded RNAs (Tuschl et al., 1999).

RISC contains single-stranded siRNAs. First, conjugation of the biotin affinity tag to the 3′ end of the target-complementary guide siRNA enabled us to affinity select sense RNA-targeting RISC, whereas 3′ biotinylation of the sense siRNA strand resulted in RISC activity in the unbound fraction. Second, single-stranded antisense RNAs were shown to reconstitute RISC. The reconstitution of RISC from single-stranded siRNA was however less effective and required 10- to 100-fold higher concentrations compared to duplex siRNA. This indicates that a specific RISC assembly pathway exists in HeLa cells, which may be bypassed by sufficiently high concentrations of single-stranded RNAs. Reconstitution of RISC from single-stranded siRNA was undetectable using *D. melanogaster* embryo lysate, which is most likely explained by the high content of 5′ to 3′ exonucleases in embryo lysate.

The size of RISC in HeLa lysate was determined by gel filtration as well as glycerol gradient ultracentrifugation after streptavidin affinity purification with 3′ biotinylated siRNA duplexes. The affinity-purified human and *D. melanogaster* RISC sediments around 90 to 160 kDa. Previously, sizes for nonaffinity-purified RISC in *D. melanogaster* systems have been reported within a range of less than 230 to 500 kDa (Hammond et al., 2000, 2001; Nykänen et al., 2001). The size differences could indicate that some RISC-associated proteins were lost during our affinity purification, but it is also conceivable that crude RISC fractions are not suitable for precise size determination of RISC because of unspecific interactions encountered in an unpurified lysate.

Two proteins of the Argonaute family, eIF2C1 and eIF2C2 (human GERp95), were identified by mass spectrometry in the affinity-purified human RISC. Argonaute proteins are a distinct class of proteins, containing a PAZ and Piwi domain (Cerutti et al., 2000) and have been implicated in many processes previously linked to posttranscriptional silencing, however only limited biochemical information is available. The first evidence that Argonaute proteins are part of RISC was provided by classical biochemical fractionation studies using dsRNA-transfected *D. melanogaster* S2 cells (Hammond et al., 2001). However, because of the high homology between the many family members of the Argonaute proteins, the mammalian ortholog of the RISC *D. melanogaster* Argonaute2 could not be predicted. More recently, the closest relative to *D. melanogaster* Argonaute2, *D. melanogaster* Argonaute1, was also shown to be required for RNAi (Williams and Rubin, 2002).

Human eIF2C2 is the ortholog of rat GERp95, which was identified as a component of the Golgi complex or the endoplasmic reticulum and copurified with intracellular membranes (Cikaluk et al., 1999). More recently, HeLa cell eIF2C2 was shown to be associated with miRNAs and

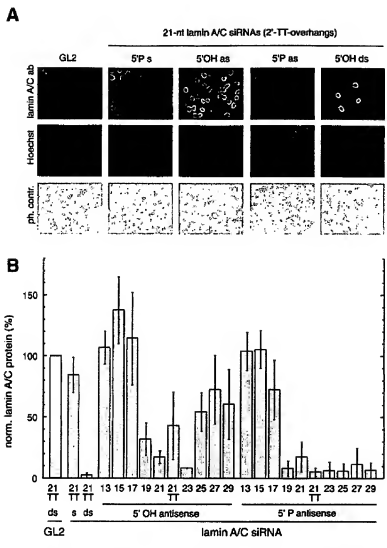

Figure 7. Single-Stranded Antisense siRNAs Mediate Gene Silencing in HeLa Cells

(A) Silencing of nuclear envelope protein lamin A/C. Fluorescence staining of cells transfected with lamin A/C-specific siRNAs and GL2 luciferase (control) siRNAs. Top row, staining with lamin A/C specific antibody; middle row, Hoechst staining of nuclear chromatin; bottom row, phase contrast images of fixed cells.

(B) Quantification of lamin A/C knockdown after Western blot analysis. The blot was stripped after lamin A/C probing and reprobed with vimentin antibody. Quantification was performed using a Lumi-Imager (Roche) and LumiAnalyst software to quantitate the ECL signals (Amersham Biosciences), differences in gel loading were corrected relative to nontargeted vimentin protein levels. The levels of lamin A/C protein were normalized to the nonspecific GL2 siRNA duplex.

components of the SMN complex, a regulator of ribonucleoprotein assembly, suggesting that eIF2C2 plays a role in miRNA precursor processing or miRNA function (Mourelatos et al., 2002). A more provocative hypothesis is that miRNAs are also in a RISC-like complex, which could potentially mediate target RNA degradation, if only perfectly matched miRNA target mRNAs existed. Sequence analysis using cloned human and mouse miRNAs, however, did not reveal the presence of such perfectly complementary sequences in the genomes (Lagos-Quintana et al., 2001). Therefore, miRNPs may only function as translational regulators of partially mismatched target mRNAs, probably by recruiting additional factors that prevent dissociation from mismatched target mRNAs.

Human eIF2C1 has not been linked to gene silencing previously, but it is more than 80% similar in sequence to eIF2C2 (Koesters et al., 1999). This similarity may indicate functional redundancy, but it is also conceivable that functional RISC may contain eIF2C1 and eIF2C2 heterodimers. The predicted molecular weight of this heterodimeric complex would be slightly larger than the observed size, but because size fractionation is based on globular shape, we cannot disregard this possibility at this time. Before reconstitution of RISC is not accomplished by using recombinant proteins, the possibility remains that the endonucleolytic activity resides in another molecule that is limited in amounts and still escapes our detection.

Single-Stranded 5′-Phosphorylated Antisense siRNAs as Triggers of Mammalian Gene Silencing

It was previously noted that introduction of sense and antisense RNAs of several hundred nucleotides in length into *C. elegans* was able to sequence specifically silence homologous genes (Guo and Kemphues, 1995). Later, it was suggested that the sense and antisense RNA preparation were contaminated with a small amount of dsRNA, which was responsible for the silencing effect and is a much more potent inducer of gene silencing (Fire et al., 1998). Most recently, it was however shown that 5′-phosphorylated antisense RNAs between 22 and 40 nt, but not sense RNAs, were able to activate gene silencing in *C. elegans*, and therefore directly contributed to initiation of gene silencing (Tijsterman et al.,

2002). The authors favored the hypothesis that gene silencing was initiated by siRNA-primed dsRNA synthesis, because 3′-blocked antisense RNAs were not functional. We have shown that modification of the 3′ ends of antisense siRNA did not interfere with reconstitution of RISC in the human system. Together, these observations suggests the driving forces for gene silencing in *C. elegans* may be predominantly dsRNA synthesis followed by Dicer cleavage, while in human and possibly also in *D. melanogaster*, RISC-specific target mRNA degradation predominates.

Targeting of endogenously expressed lamin A/C by transfection of duplex siRNA into HeLa cells was the first reported example of siRNA-induced gene silencing (Elbashir et al., 2001a). Lamin A/C protein was drastically reduced by a lamin A/C-specific siRNA duplex within two days posttransfection, while unspecific siRNA duplexes showed no effect. At the time, transfection of nonphosphorylated sense or antisense siRNA did not reveal a substantial effect on lamin A/C levels (Elbashir et al., 2001a); although more recently, a minor reduction upon antisense siRNA transfection was noticed when similar concentrations of antisense siRNA were delivered as described here (Elbashir et al., 2002). However, the effect was not interpreted as RISC-specific. Assaying 5′-phosphorylated antisense siRNAs revealed a substantial increase in lamin A/C silencing, probably because 5′-phosphorylated siRNAs are more stable or enter RISC more rapidly, or because the 5′ end of transfected single-stranded siRNA may be less rapidly phosphorylated in the cell in comparison to duplex siRNAs.

Finally, it should be noted that HeLa cells are generally poor in nucleases and represent one of the preferred mammalian systems for studying RNA processing or transcription reactions in vivo and in vitro. It remains to be tested if 5′-phosphorylated single-stranded antisense siRNAs are suitable to knockdown gene expression in other cell types or tissues with a different content of nucleases. The general silencing ability of various cell types may also depend on the relative levels of siRNA/miRNA-free eIF2C1 and eIF2C2 proteins capable of associating with exogenously delivered siRNAs. From a technical perspective, the sensitivity of single-stranded siRNA toward nucleases present in tissue culture medium and serum may complicate the application of single-stranded siRNAs. However, chemical strategies to improve nuclease resistance of single-stranded RNA are available.

In summary, single-stranded 5′-phosphorylated antisense siRNAs of 19 to 29 nt in size broaden the use of RNA molecules for gene silencing because they can enter the mammalian RNAi pathway in vitro as well as in vivo through reconstitution of RISC. Human eIF2C1 and eIF2C2 seem to play a critical role in this process. Considering the feasibility of modulating the stability and uptake properties of single-stranded RNAs, 5′-phosphorylated single-stranded antisense siRNAs may further expand the utility of RNAi-based gene silencing technology as tool for functional genomics as well as therapeutic applications.

Experimental Procedures

siRNA Synthesis and Biotin Conjugation

siRNAs were chemically synthesized using RNA phosphoramidites (Proligo, Hamburg, Germany) and deprotected and gel purified as described previously (Elbashir et al., 2001a). Some of the RNAs were also synthesized using 2′-O-ACE-RNA phosphoramidites (Dharmacon, Lafayette, Co, USA) and deprotected as described (Scaringe, 2001). 5′ aminolinkers were introduced by coupling MMT-C6-aminolinker phosphoramidite (Proligo, Hamburg), 3′ C7-aminolinkers were introduced by assembling the oligoribonucleotide chain on 3′-amino-modifier (TFA) C7 lcaa control pore glass support (Chemgenes, MA, USA). The sequences for GL2 and RL luciferase siRNAs were as described (Elbashir et al., 2001a). The 29 nt antisense siRNA directed against lamin A/C was 5′ UGUUCUUCUGGAAGUCCAGUUCCUC CUUC, shorter antisense RNAs are derived by truncations from the 3′ end. If 5′ phosphates were to be introduced, 50 to 100 nmoles of synthetic siRNAs were treated with T4 polynucleotide kinase (300 μl reaction, 2.5 mM ATP, 70 mM Tris-HCl, [pH 7.6], 10 mM $MgCl_2$, 5 mM DTT, 30 U T4 PNK, New England Biolabs, 45 min, 37°C) followed by ethanol precipitation.

3′ Terminal ^{32}pCp labeling (Figure 2) was performed in a 30 μl reaction (17 μM siRNA, 0.5 μM ^{32}pCp (110 TBq/mmol), 15% DMSO, 20 U T4 RNA ligase, NEB, and 1× NEB-supplied reaction buffer) for 1.5 hr at 37°C, and gel purified. One half of the pCp-labeled RNA was dephosphorylated (25 μl reaction, 500 U alkaline phosphatase, Roche, and Roche-supplied buffer, 30 min, 50°C), followed by phenol/chloroform extraction and ethanol precipitation. Half of this reaction was 5′-phosphorylated (20 μl reaction, 2 units T4 polynucleotide kinase, NEB, 10 mM ATP, NEB-supplied buffer, 60 min, 37°C). A quarter of the initial pCp-labeled siRNA was also 5′-phosphorylated (10 μl reaction, 10 units 3′ phosphatase-free T4 polynucleotide kinase, Roche, 10 mM ATP, Roche-supplied buffer, 3 min, 37°C).

For conjugation to biotin, 20 to 65 nmoles of fully deprotected aminolinker-modified siRNA were dissolved in 100 μl of 100 mM sodium borate buffer (pH 8.5) and mixed with a solution of 1 mg of EZ-Link NHS-PC-LC-Biotin (Pierce, IL, USA) in 100 μl of anhydrous dimethylformamide. The solution was incubated for 17 hr at 25°C in the dark. Subsequently, siRNAs were precipitated by the addition of 60 μl 2 M sodium acetate (pH 6.0) and 1 ml ethanol. The RNA pellet was collected by centrifugation and biotin-conjugated siRNA was separated from nonreacted siRNA on a preparative denaturing 18% acrylamide gel (40 cm length) in the dark. The RNA bands were visualized by 254 nm UV shadowing and minimized exposure time. The bands were excised, and the RNA was eluted overnight in 0.3 M NaCl at 4°C and recovered by ethanol precipitation. siRNA duplexes were formed as previously described (Elbashir et al., 2002).

Preparation of S100 Extract from HeLa Cells

Cytoplasm from HeLa cells adapted to grow at high density by Computer Cell Culture, Mons, Belgium, was prepared following the Dignam protocol for isolation of HeLa cell nuclei (Dignam et al., 1983). The cytoplasmic fraction was supplemented with KCl, $MgCl_2$, and glycerol to final concentrations of 100 mM, 2 mM, and 10%, respectively. At this stage, the extract can be stored frozen at −70°C after quick-freezing in liquid nitrogen without loss of activity. S100 extract was prepared by ultracentrifugation at 31.500 rpm for 60 minutes at 4°C using a Sorvall T-865 rotor. The protein concentration of HeLa S100 extract varied between 4 to 5 mg/ml as determined by Bradford assay.

Affinity Purification of RISC with 3′ Biotinylated siRNA Duplexes

For affinity purification of siRNA-associated protein complexes from HeLa S100 extract, 10 nM of a 3′ double-biotinylated siRNA duplex was incubated in 0.2 mM ATP, 0.04 mM GTP, 10 U/ml RNasin, 6 μg/ml creatine kinase, and 5 mM creatine phosphate in 60% S100 extract at 30°C for 60 min and gentle rotation. Thereafter, 1 ml slurry of immobilized Neutravidin Biotin Binding Protein (Pierce, IL, USA) was added per 50 ml of reaction solution and the incubation was continued for another 120 min at 30°C with gentle rotation. The Neutravidin beads were then collected at 2000 rpm for 2 minutes at 4°C in a Heraeus Megafuge 1.0 R centrifuge using a swinging bucket rotor type 2704. Effective capturing of RISC components after affinity selection was confirmed by assaying the supernatant for residual RISC activity with and without supplementing fresh siRNA duplexes. The collected Neutravidin beads were washed with 10 volumes of buffer A relative to the bead volume (30 mM HEPES, [pH 7.4], 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, and 10% glycerol)

followed by washing with 5 volumes of buffer B (same as buffer A with only 3% glycerol content). The beads were transferred to a 0.8 × 4 cm Poly-Prep chromatography column (BioRad; CA, USA) by resuspending in 3 volumes of buffer B at 4°C, followed by 10 volumes of washing with buffer B. Washing of the beads was continued by 10 volumes of buffer B increased to 300 mM KCl. The column was then reequilibrated with regular buffer B. To recover native siRNA-associated complexes, the column was irradiated in the cold room by placing it at a 2 cm distance surrounded by four 312 nm UV lamps (UV-B tube, 8 W, Herolab, Germany) for 30 minutes. To recover the photocleaved siRNP solution, the column was placed into a 50 ml Falcon tube and centrifuged at 2000 rpm for 1 minute at 4°C using again the 2704 rotor. For full recovery of siRNPs, the beads were once again resuspended in buffer B followed by a second round of UV treatment for 15 minutes. Both eluates were pooled and assayed for target RNA degradation.

Target RNA Cleavage Assays

Cap-labeled target RNA of 177 nt was generated as described (Elbashir et al., 2001c), except that his-tagged guanylyl transferase was expressed in *E. coli* from a plasmid generously provided by J. Wilusz and purified to homogeneity. If not otherwise indicated, 5′-phosphorylated siRNA or siRNA duplex was preincubated in supplemented HeLa S100 extract at 30°C for 15 min prior to addition of cap-labeled target RNA. After addition of all components, final concentrations were 100 nM siRNA, 10 nM target RNA, 1 mM ATP, 0.2 mM GTP, 10 U/ml RNasin, 30 μg/ml creatine kinase, 25 mM creatine phosphate, and 50% S100 extract. Incubation was continued for 2.5 hr. siRNA-mediated target RNA cleavage in *D. melanogaster* embryo lysate was performed as described (Zamore et al., 2000). Affinity-purified RISC in buffer B was assayed for target RNA cleavage without preincubation nor addition of extra siRNA (10 nM target RNA, 1 mM ATP, 0.2 mM GTP, 10 U/ml RNasin, 30 μg/ml creatine kinase, 25 mM creatine phosphate, and 50% RISC in buffer B). Cleavage reactions were stopped by the addition of 8 vols of proteinase K buffer (200 mM Tris-HCl [pH 7.5], 25 mM EDTA, 300 mM NaCl, and 2% w/v SDS. Proteinase K, dissolved in 50 mM Tris-HCl [pH 8.0], 5 mM $CaCl_2$, and 50% glycerol, was added to a final concentration of 0.6 mg/ml and processed as described (Zamore et al., 2000). Samples were separated on 6% sequencing gels.

Analytical Gel Filtration

UV-eluates in buffer B were fractionated by gel filtration using a Superdex 200 PC 3.2/30 column (Amersham Biosciences) equilibrated with buffer A on a SMART system (Amersham Biosciences). Fractionation was performed by using a flow rate of 40 μl/minute and collecting 100 μl fractions. Fractions were assayed for specific target RNA cleavage. Size calibration was performed using molecular size markers thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and BSA (66 kDa) (Amersham Biosciences).

Glycerol Gradient Sedimentation

UV-eluates were layered on top of 4 ml linear 5%–20% (w/w) glycerol gradient adjusted to 30 mM HEPES, [pH 7.4], 100 mM KCl, 2 mM $MgCl_2$, and 0.5 mM DTT. Centrifugation was performed at 35,000 rpm for 14.5 hr at 4°C using a Sorvall SW 60 rotor. Twenty fractions of 0.2 ml volume were removed sequentially from the top and 15 μl aliquots were used to assay for target RNA cleavage. For large-scale protein purification, 400 ml of HeLa S100 extract was used. UV-eluates were layered on top of a 12.8 ml glycerol gradient. Ultracentrifugation was performed at 37,000 rpm for 21 hr at 4°C using a Centrikon TST 41.14 rotor. Twenty fractions of 0.64 ml volume were sequentially removed from the top.

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Note Added in Proof

Targeted mRNA degradation by single-stranded antisense RNA and involvement of eIF2C2 in HeLa cells was independently observed by P.D. Zamore and colleagues (Hutvágner, G., and Zamore, P.D. [2002]. A microRNA in a multiple-turnover RNAi enzyme complex. Science, in press. Published online August 1, 2002. 10.1126/science.1073827; Schwarz, D., Hutvágner, G., Haley, G., and Zamore, P.D. [2002] siRNAs function as guides, not primers, in the RNAi pathway in *Drosphila* and human cells. Molecular Cell, in press).